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Novel Enzyme

The present invention relates to a novel protein, in particular mutant luciferase enzymes which have increased thermostability as compared to the corresponding wild type enzyme, to the use of these enzyme in assays and to test kits containing them.

Firefly luciferase catalyses the oxidation of luciferin in the presence of ATP, Mg^{2+} and molecular oxygen with the resultant production of light. This reaction has a quantum yield of about 0.88. The light emitting property has led to its use in a wide variety of luminometric assays where ATP levels are being measured. Examples of such assays include those which are based upon the described in EP-B-680515 and WO 96/02665.

Luciferase is obtainable directly from the bodies of insects, in particular beetles such as fireflies or glow-worms. Particular species from which luciferases have been obtained include the Japanese GENJI or KEIKE fireflies, *Luciola cruciata* and *Luciola lateralis*, the East European firefly *Luciola mingrelica* , the North American firefly *Photinus pyralis* and the glow-worm *Lampyris noctiluca*.

However, since many of the genes encoding these enzymes have been cloned and sequenced, they may also be produced using recombinant DNA technology. Recombinant DNA sequences encoding the enzymes are used to transform microorganisms such as *E. coli* which then express the desired enzyme product.

The heat stability of wild and recombinant type luciferases is such that they lose activity quite rapidly when exposed to temperatures in excess of about 30°C, particularly over 35°C. This instability causes problems when the enzyme is used or stored at high ambient temperature, or if the assay is effected

under high reaction conditions, for example in order to increase reaction rate.

Mutant luciferases having increased thermostability are known from EP-A-524448 and WO/95/25798. The first of these describes a mutant luciferase having a mutation at position 217 in the Japanese firefly luciferase, in particular by replacing a threonine residue with an isoleucine residue. The latter describes mutant luciferases having over 60% homology to luciferase from *Photinus pyralis*, *Luciola mingrelica*, *Luciola cruciata* or *Luciola lateralis* but in which the amino acid residue corresponding to residue 354 of *Photinus pyralis* or 356 of the *Luciola* species is mutated such that it is other than glutamate.

The applicants have found yet a further mutation which can bring about increased thermostability and which may complement the mutations already known in the art.

The present invention provides a protein having luciferase activity and at least 60% homology to luciferase from *Photinus pyralis*, *Luciola mingrelica*, *Luciola cruciata* or *Luciola lateralis* enzyme wherein in the sequence of the enzyme, at least one of

(a) the amino acid residue corresponding to residue 214 in *Photinus pyralis* luciferase and to residue 216 of *Luciola mingrelica*, *Luciola cruciata* or *Luciola lateralis* luciferase is other than threonine; or

(b) the amino acid residue corresponding to residue 232 in *Photinus pyralis* luciferase and to residue 234 of *Luciola mingrelica*, *Luciola cruciata* or *Luciola lateralis* luciferase is other than isoleucine; or

(c) amino acid residue corresponding to residue 295 in *Photinus pyralis* luciferase and to residue 297 of *Luciola mingrelica*,

Luciola cruciata or *Luciola lateralis* luciferase is other than phenylalanine;
and the luciferase enzyme has increased thermostability as compared to the wild-type luciferase.

The sequences of all the various luciferases show that they are highly conserved having a significant degree of homology between them. This means that corresponding regions among the enzyme sequences are readily determinable by examination of the sequences to detect the most similar regions, although if necessary commercially available software (e.g. "Bestfit" from the University of Wisconsin Genetics Computer Group; see Devereux et al (1984) Nucleic Acid Research 12: 387-395) can be used in order to determine corresponding regions or particular amino acids between the various sequences. Alternatively or additionally, corresponding acids can be determined by reference to L. Ye et al., Biochim. Biophys Acta 1339 (1997) 39-52.

With respect to the possible change of the amino acid residue corresponding to residue 214 in *Photinus pyralis* luciferase and to residue 216 of *Luciola mingrelica*, *Luciola cruciata* or *Luciola lateralis* luciferase, the polar amino acid threonine is suitably replaced with a non polar amino acid such as alanine, glycine, valine, lecine, isoleucine, proline, phenylalanine, methionine, tryptophan or cysteine. A particularly preferred substitution for the threonine residue corresponding to residue 214 in *Photinus pyralis* luciferase and to residue 216 of *Luciola mingrelica*, *Luciola cruciata* or *Luciola lateralis* luciferase at group is alanine.

As regards the the possible change of the amino acid residue corresponding to residue 232 in *Photinus pyralis* luciferase and to residue 234 of *Luciola mingrelica*, *Luciola cruciata* or

Luciola lateralis luciferase, the nonpolar amino acid isoleucine is suitably replaced with a different non polar amino acid such as alanine, glycine, valine, lecine, proline, phenylalanine, methionine, tryptophan or cysteine. A particularly preferred substitution for the isoleucine residue corresponding to residue 232 in *Photinus pyralis* luciferase and to residue 234 of *Luciola mingrelica*, *Luciola cruciata* or *Luciola lateralis* luciferase at group is alanine.

Finally, changes of the amino acid residue corresponding to residue 295 in *Photinus pyralis* luciferase and to residue 297 of *Luciola mingrelica*, *Luciola cruciata* or *Luciola lateralis* luciferase, the non-polar amino acid phenylalanine is suitably replaced with a different non polar amino acid, other than leucine, such as alanine, glycine, valine, isoleucine, proline, methionine, tryptophan or cysteine. A particularly preferred substitution for the phenylalanine residue corresponding to residue 214 in *Photinus pyralis* luciferase and to residue 216 of *Luciola mingrelica*, *Luciola cruciata* or *Luciola lateralis* luciferase at group is leucine.

Proteins of the invention suitably have more than one such mutation, and preferably all three of the mutations described above.

Other mutations may also be present in the enzyme. For example, in a preferred embodiment, the protein also has the amino acid at position corresponding to amino acid 354 of the *Photinus pyralis* luciferase (356 in Luciola luciferase) changed from glutamate, in particular to an amino acid other than glycine, proline or aspartic acid. Suitably, the amino acid at this position is tryptophan, valine, leucine, isoleucine are asparagine, but most preferably is lysine or arginine. This mutation is described in WO 95/25798.

In an alternative preferred embodiment, the protein also has the amino acid at the position corresponding to amino acid 217 in Luciola luciferase (215 in *Photinus pyralis*) changed to a hydrophobic amino acid in particular to isoleucine, leucine or valine as described in EP-A-052448.

Yet further mutations may occur in the luciferase in order to further enhance the thermostability. Specific examples of such further mutations include

(i) mutation at the amino acid residue corresponding to amino acid 14 of the *Photinus pyralis* luciferase (16) in Luciola luciferase) changed from phenylalanine to a different amino acid, in particular to a different nonpolar amino acid such as alanine, valine, leucine, isoleucine, proline, methionine or tryptophan, preferably alanine; and/or

(ii) mutation at the amino acid residue corresponding to amino acid 35 of the *Photinus pyralis* luciferase (37) in Luciola luciferase) which is changed from leucine to a different amino acid, in particular to a different nonpolar amino acid such as alanine, valine, phenylalanine, isoleucine, proline, methionine or tryptophan, and preferably alanine.

Proteins of the invention include both wild-type and recombinant luciferase enzymes. They have at least 60% homology to the sequences of *Photinus pyralis*, *Luciola mingrelica*, *Luciola cruciata* or *Luciola lateralis* enzyme in the sense that at least 60% of the amino acids present in the wild-type enzymes are present in the proteins of the invention. Such proteins can have a greater degree of homology, in particular at least 70%, more preferably at least 80% and most preferably at least 90% to the wild-type enzymes listed above. Homologous proteins are of this type include allelic variants, proteins from other insect species as well as

recombinantly produced enzymes. They can be readily identified in that they are encoded by nucleic acids which hybridise with sequences which encode wild-type enzymes under stringent hybridisation conditions. Such conditions would be well understood by the person skilled in the art, and are exemplified for example in Sambrook et al. (1989) Molecular Cloning, Cold Spring Harbor Laboratory Press).

Particular examples of proteins of the invention are wild-type luciferase sequence with the mutations as outlined above.

The invention further provides nucleic acids which encode the luciferases as described above. Suitably, the nucleic acids are based upon wild-type sequences which are well known in the art. Suitable mutation to effect the desired mutation in the amino acid sequence would be readily apparent, based upon a knowledge of the genetic code.

The nucleic acids of the invention are suitably incorporated into an expression vector such as a plasmid under the control of control elements such as promoters, enhancers, terminators etc. These vectors can then be used to transform a host cell, for example a prokaryotic or eukaryotic cell, but in particular a prokaryotic cell such as *E. coli* so that the cell expresses the desired luciferase enzyme. Culture of the thus transformed cells using conditions which are well known in the art will result in the production of the luciferase enzyme which can then be separated from the culture medium. Vectors, transformed cells and methods of producing enzyme by culturing these cells all form further aspects of the invention.

The *Photinus pyralis* T214A mutant luciferase was created by random mutagenesis as described hereinafter. It was found

that the T214A single point mutation has greater thermostability than wild type luciferase.

Two new triple mutant luciferases: E354K/T214A/A215L and E354K/T214A/I232A were also prepared and these also have exhibited greater thermostability.

Particular examples of mutant enzymes of *Photinus pyralis* which fall within the scope of the invention include the following:
I232A/E354K
T214A/I232A/E354K
A215L/I232A/E354K
T214A/I232A/E354K/A215L
I232A/E354K/T214A/F295L
I232A/E354K/T214A F295L/F14A/L35A
I232A/E354K/T214A/F295L/F14A/L35A/A215L

or equivalents of any of these when derived from the luciferases of other species.

The mutations for the creation of the triple mutant were introduced to the luciferase gene on plasmid pET23 by site-directed mutagenesis, (PCR). The oligonucleotides added to the PCR reaction in order to effect the relevant mutations are shown Figure

It has been reported previously that the effect of point mutations at the 354 and 215 positions are additive. This invention provides the possibility of combining three or more such mutations to provide still greater thermostability.

Thermostable luciferase of the invention will advantageously be employed in any bioluminescent assay which utilises the luciferase/luciferin reaction as a signalling means. There

are many such assays known in the literature. The proteins may therefore be included in kits prepared with a view to performing such assays, optionally with luciferin and any other reagents required to perform the particular assay.

The invention will now be particularly described by way of example with reference to the accompanying diagrammatic drawings in which:

Figure 1 illustrates the plasmids used in the production of mutants in accordance with the invention;

Figure 2 shows the results of heat inactivation studies on luciferases including luciferases of the invention;

Figure 3 shows the results of thermostability experiments on various luciferase mutants; and

Figure 4 is a graph showing thermostability data at 45°C for selected mutants.

Example 1

Identification of Thermostable Mutant Luciferase

The error-prone PCR was based on the protocol devised by Fromant *et al.*, Analytical Biochemistry, 224, 347-353 (1995).

The dNTP mix in this reaction was:

35mM dTTP

12.5mM dGTP

22.5mM dCTP

14mM dATP

The PCR conditions were:

0.5 µl (50ng) plasmid pPW601a J54*

5.0 µl 10x KCl reaction buffer

1 µl W56-60 pmoles of each primer + 1 µl W57

1 µl Biotaq ™ polymerase (5U)

2 µl dNTPs (see above)

1.76 µl $MgCl_2$ (50 mM stock)

1 µl $mNCl_2$ (25mM stock) [final concentration in reaction = 3.26mM]

36.7 µl dH_2O

(*Plasmid pPW601aJ54 is a mutated version of pPW601a (WO where an NdeI site has been created within the 3 bases prior to the ATG start codon. This allows for easy cloning from pPW601a into the pET23 vector.

+Primer sequences:

W56:

5' - AAACAGGGACCCATATGGAAGACGC - 3'

W57:

5' - AATTAACTCGAGGAATTTCGTCATCGCTGAATACAG - 3')

Cycling parameters were:

94 °C-5 min

Then 12 x cycles of: 94 °C-30s

55 °C-30s

72 °C-5min

72 °C-10 min

The PCR products were purified from the reaction mix using a Clontech Advantage ™ PCR-pure kit. An aliquot of the purified products was then digested with the restriction enzymes NdeI and XhoI. The digested PCR products were then "cleaned up"

with the Advantage kit and ligated into the vector pET23a which had been digested with the same enzymes.

Ligation conditions:

4µl pET23a (56ng)
5µl PCR products (200ng)
3µl 5x Gibco BRL ligase reaction buffer
1µl Gico BRL ligase (10U)
2µl dH_2O

The ligation was carried out overnight at 16°C.

The ligated DNAs were then purified using the Advantage™ kit and then electroporated into electrocompetent *E. coli* HB101 cells (1mm cuvettes, 1.8 Kv).

Eleven electroporations were performed and the cells were then added to 40 ml of TY broth containing 100µg/ml ampicillin. The cells were then grown overnight at 37°C. The entire 50ml of culture grown overnight was used to purify plasmid DNA. This is the library.

Screening the library

An aliquot of the plasmid library was used to electroporate *E. coli* BL21 DE3 cells. These cells were then plated onto LB agar containing 50µg/ml ampicillin and grown overnight at 37°C.

The next day, colonies were picked and patched onto nylon filters on LB agar + amp plates and growth continued overnight at 37°C. The next day, filters were overlaid with a solution of luciferin - 500µM in 100mM sodium citrate pH5.0. The

patches were then viewed in a darkroom. One colony/patch was picked from 200 for further analysis.

Characterisation of the thermostable mutant

The *E. coli* clone harbouring the mutant plasmid was isolated. Plasmid DNA was prepared for ABI sequencing. The entire open reading frame encoding luciferase was sequenced using 4 different oligonucleotide primers. Sequencing revealed a single point mutation at nt 640 (A → G). Giving a codon change of ACT (T) to GCT (A) at amino acid position 214.

Example 2

Preparation of Triple Mutant Enzyme

A mutagenic oligonucleotide was then used to create this same mutation in pMOD1 (A215L/E354K) to create a triple mutant pMOD2 (A215L/E354K/T214A). This mutation also creates a unique SacI/SstI site in pMOD1.

Example 3

Preparation of further triple mutant enzyme

The following primers were used to create the triple mutant T214A/I232A/E354K using a standard PCR reaction and with the pET23 plasmid with the T214A mutation as template:

CTGATTACACCCAAGGGGGATG	E354K-sense
CATCCCCCTTGGGTGTAATCAG	E354K-antisense
GCAATCAAATCGCTCCGGATACTGC	I232A-sense
GCAGTATCCGGAGCGATTTGATTGC	I232A-antisense.

Example 3

Identification of thermostable 295 mutant

The F295 mutant was created using the error-prone PCR method described by Fromant et al., Analytical Biochemistry, vol 224, 347-353 (1995). The PCR conditions used were as follows:

0.5 μl (50 ng) plasmid pET23
5.0 μl 10x KCI reaction buffer
1 μl primer 1 - 60 pmoles of each primer
1 μl primer 2
1 μl Biotaq™ polymerase (5U)
2 μl dNTPs, in mixture 35 mM dTTP, 12.5 mM dGTP, 22.5 mM dCTP, 14 mM dATP
1.76 μl $MgCl_2$ (50 mM stock)
1 μl $MnCl_2$ (25 mM stock) [final concentration in reaction = 3.26 mM]
36.7 μl dH_2O
Primer 1 = 5' - AAACAGGGACCCATATGGAAGACGC - 3'
Primer 2 = 5' - AATTAACTCGAGGAATTTCGTCATCGCTGAATACAG - 3'

The cycling parameters were:
94°C for 5 min
15 cycles of: 30 s @ 94°C
30 s @ 55°C
5 min @ 72°C
then 10 min at 72°C

The PCR products were purified from the reaction mix using a Clontech Advantage™ PCR-Pure kit. An aliquot of the purified products was then digested with the restriction enzymes NdeI and XhoI. The digested PCR products were then "cleaned up" with the Advantage™ kit and ligated into the vector pET23a, which had been digested with the same enzymes.

The ligation condition were as follows:

56 ng pET23a
200 ng PCR products
3 μl 5x Gibco BRL ligase reaction buffer
1μl Gibco BRL ligase (10U)
volume made up to 10 μl with dH_2O

The ligation was carried out overnight at 16°C.

The ligated DNAs were then purified using the Advantage™ kit and then electroporated into electrocompetent *Escherichia coli* DH5α cells (1mm cuvettes, 1.8kV). 1ml of SOC broth was added to each electroporation and the cells allowed to recover and express antibiotic resistance genes encoded by the plasmid. Aliquots of the library were inoculated onto LB agar containing 50 μg/ml ampicillin and the bacteria were grown overnight at 37°C. Nylon filter discs were then overlaid onto the agar plates and the colonies transferred to fresh plates. The original plates were left at room temperature for the colonies to re-grow. The plates with the nylon filters were incubated at 42°C for 2 h before plates were sprayed with 500μM luciferin in 100mM citrate buffer pH5.0 and viewed in a darkroom.

Three thermostable colonies were selected on the basis that they still glowed after 2 h at 42°C. Plasmid DNA was isolated from these clones and sequenced, and this revealed the F295L mutation in each case.

Example 4

Other mutants of the invention were produced by PCR using appropriate combinations of the oligonucleotides listed above as well as the following:

GAAAGGCCCGGCACCAGCCTATCCTCTAGAGG F14A-sense

CCTCTAGCGGATAGGCTGGTGCCGGGCCTTTC F14A-antisense

GAGATACGCCGCGGTTCCTGG L35A-sense

CCAGGAACCGCGGCGTATCTC L35A-antisense

Example 5

Purification of luciferase and heat inactivation studies.

Cells expressing the recombinant mutant luciferases were cultured, disrupted and extracted as described in WO 95/25798 to yield cell free extracts of luciferase.

Eppendorf tubes containing the cell free extracts were incubated generally at 40°C unless otherwise stated. Purified preparations of wild type luciferases (for comparative purposes were incubated in thermostability buffer comprising 50mM potassium phosphate buffer pH7.8 containing 10% saturated ammonium sulphate, 1mM dithiothreitol and 0.2% bovine serum albumin (BSA). At set times a tube was removed and cooled in an ice/water bath prior to assay with remaining assayed activity being calculated as a percentage of the initial activity or relative bioluminesce.

The results are illustrated in Figures 2 and 3 hereinafter. It can be seen from Figure 2 that luciferase mutants of the invention have improved thermostability than the previously known mutants.

The dramatic increase in stability over wild-type luciferase (RWT) is clear from Figure 3.

Claims

1. A protein having luciferase activity and at least 60% homology to luciferase from *Photinus pyralis*, *Luciola mingrelica*, *Luciola cruciata* or *Luciola lateralis* enzyme wherein in the sequence of the enzyme, at least one of
(a) the amino acid residue corresponding to residue 214 in *Photinus pyralis* luciferase and to residue 216 of *Luciola mingrelica*, *Luciola cruciata* or *Luciola lateralis* luciferase is other than threonine; or
(b) the amino acid residue corresponding to residue 232 in *Photinus pyralis* luciferase and to residue 234 of *Luciola mingrelica*, *Luciola cruciata* or *Luciola lateralis* luciferase is other than isoleucine; or
(c) amino acid residue corresponding to residue 295 in *Photinus pyralis* luciferase and to residue 297 of *Luciola mingrelica*, *Luciola cruciata* or *Luciola lateralis* luciferase is other than phenylalanine;
and the luciferase enzyme has increased thermostability as compared to the wild-type luciferase.

2. A protein according to claim 1 wherein the amino acid residue corresponding to residue 214 in *Photinus pyralis* luciferase and to residue 216 of *Luciola mingrelica*, *Luciola cruciata* or *Luciola lateralis* luciferase is alanine.

3. A protein according to claim 1 or claim 2 wherein the amino acid residue corresponding to residue 232 in *Photinus pyralis* luciferase and to residue 234 of *Luciola mingrelica*, *Luciola cruciata* or *Luciola lateralis* luciferase is alanine.

4. A protein according to any one of claims 1 to 3 wherein the amino acid residue corresponding to residue 295 in *Photinus*

pyralis luciferase and to residue 297 of *Luciola mingrelica*, *Luciola cruciata* or *Luciola lateralis* luciferase is leucine.

5. A protein according to any one of the preceding claims wherein the amino acid at position corresponding to amino acid 354 of the *Photinus pyralis* luciferase (356 in Luciola luciferase) is other than glutamate.

6. A protein according to claim 5 wherein the amino acid at position corresponding to amino acid 354 of the *Photinus pyralis* luciferase (356 in Luciola luciferase) is lysine or arginine.

7. A protein according to any one of the preceding claims wherein the amino acid at the position corresponding to amino acid 217 in Luciola luciferase (215 in *Photinus pyralis*) is a different hydrophobic amino acid.

8. A protein according to claim 7 wherein the amino acid at the position corresponding to amino acid 217 in Luciola luciferase (215 in *Photinus pyralis*) is isoleucine, leucine or valine.

9. A protein according to any one of the preceding claims which includes one or more of the following:
(i) mutation at the amino acid residue corresponding to amino acid 14 of the *Photinus pyralis* luciferase (16) in Luciola luciferase) changed from phenylalanine to a different amino acid, and/or
(ii) mutation at the amino acid residue corresponding to amino acid 35 of the *Photinus pyralis* luciferase (37) in Luciola luciferase) which is changed from leucine to a different amino acid.

10. A protein according to any one of the preceding claims which is a modified wild-type luciferase.

11. A nucleic acid which encodes a luciferase according to any one of the preceding claims.

12. A nucleic acid according to claim 11 of SEQ ID NO 1 or variants thereof.

13. A vector comprising a nucleic acid according to claim 11 or 12.

14. A cell transformed with a vector according to claim 13.

15. A method of producing a protein according to any one of claims 1 to 10, which method comprises culture of a cell according to claim 14.

16. The use of a protein according to any one of claims 1 to 10 in a bioluminescent assay.

17. A kit comprising a protein according to any one of claims 1 to 10.

18. A kit according to claim 17 which further comprises luciferin.

Abstract

A protein having luciferase activity and at least 60% homology to luciferase from *Photinus pyralis*, *Luciola mingrelica*, *Luciola cruciata* or *Luciola lateralis* enzyme wherein in the sequence of the enzyme, at least one of

(a) the amino acid residue corresponding to residue 214 in *Photinus pyralis* luciferase and to residue 216 of *Luciola mingrelica*, *Luciola cruciata* or *Luciola lateralis* luciferase is other than threonine; or

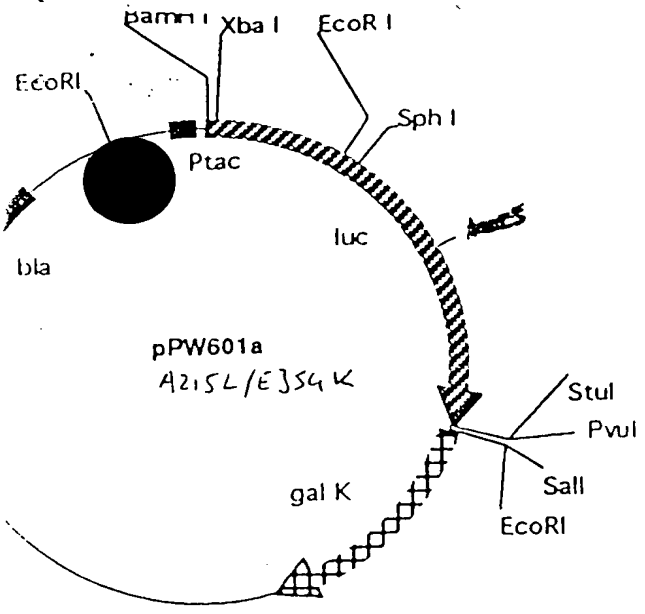
(b) the amino acid residue corresponding to residue 232 in *Photinus pyralis* luciferase and to residue 234 of *Luciola mingrelica*, *Luciola cruciata* or *Luciola lateralis* luciferase is other than isoleucine; or

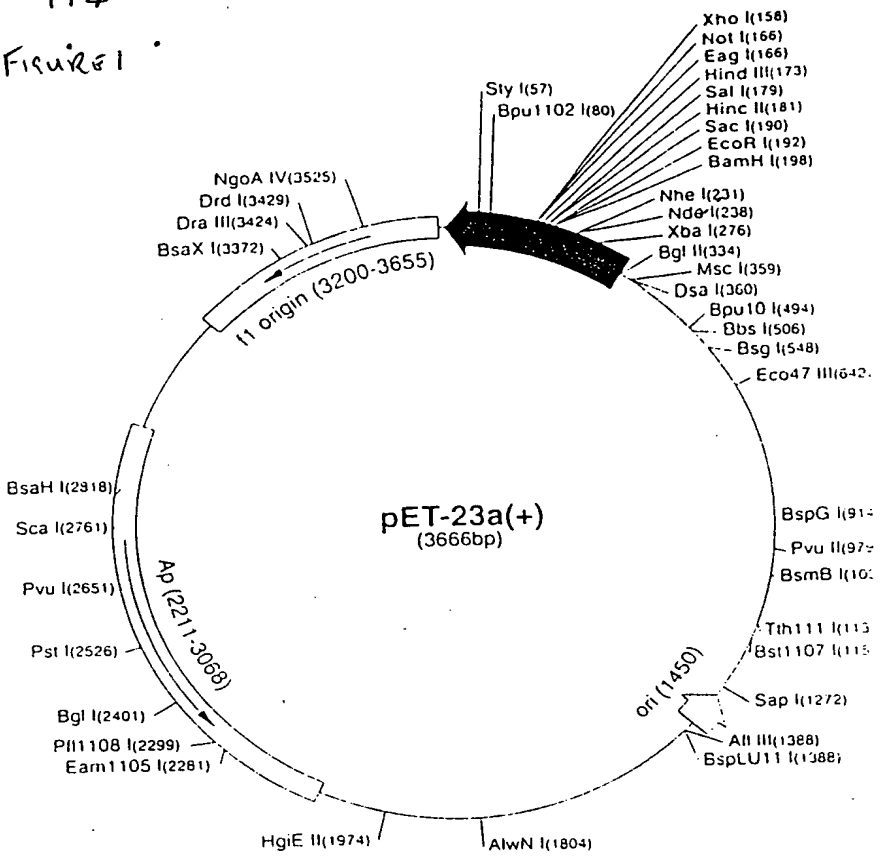
(c) amino acid residue corresponding to residue 295 in *Photinus pyralis* luciferase and to residue 297 of *Luciola mingrelica*, *Luciola cruciata* or *Luciola lateralis* luciferase is other than phenylalanine;

and the luciferase enzyme has increased thermostability as compared to the wild-type luciferase.

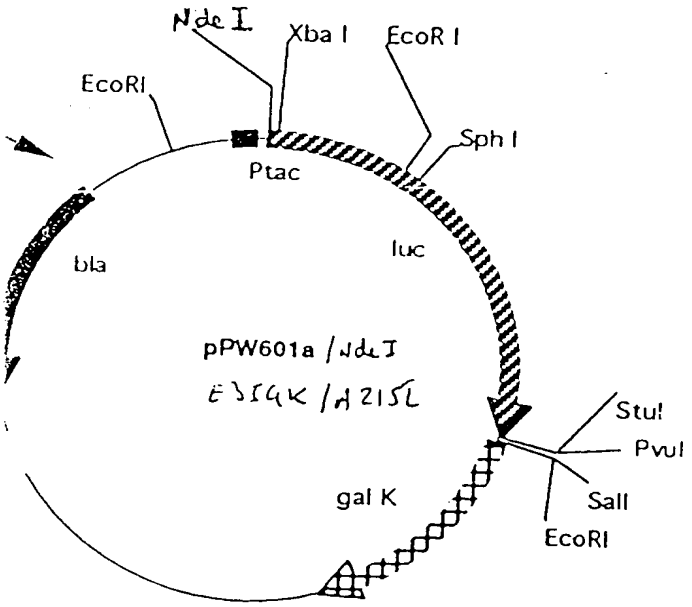
These enzymes, which optionally have additional mutations, will be useful in assays.

Figure 1





site directed mutagenesis
BamHI → NdeI



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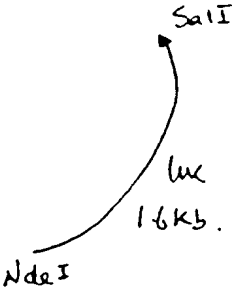

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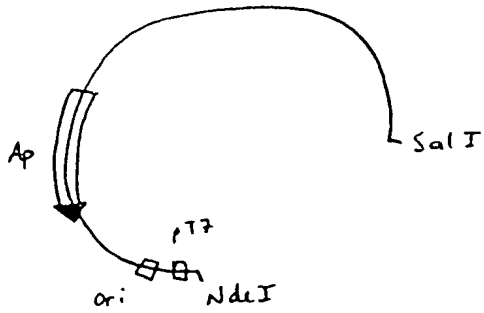


ligate.

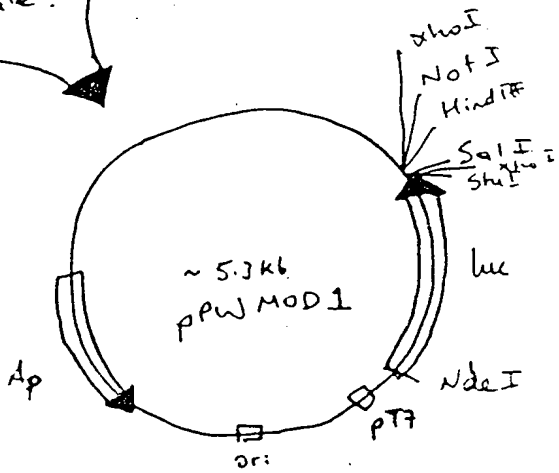


FIGURE 2

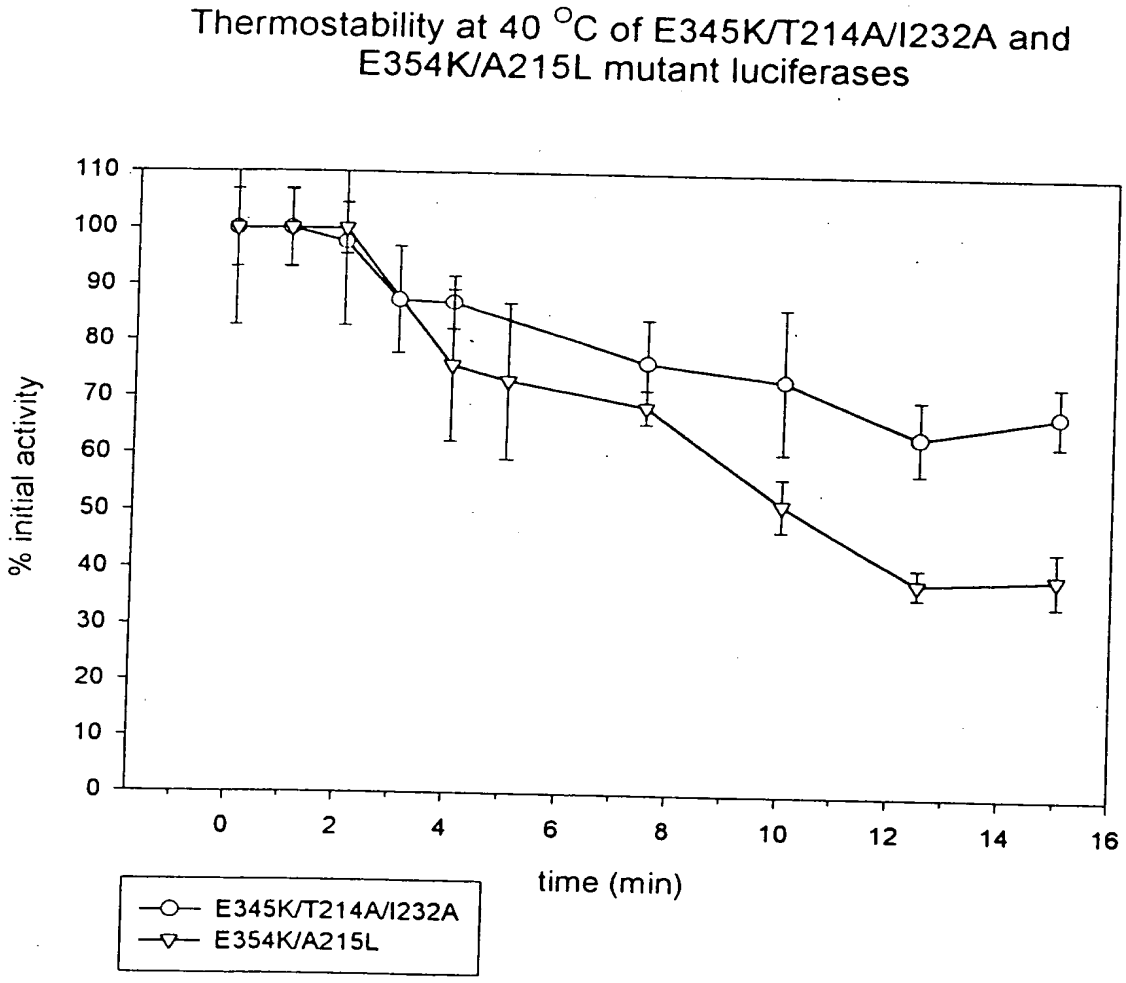
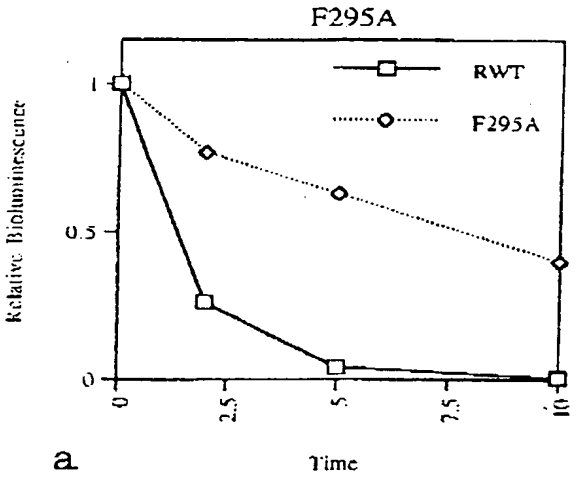
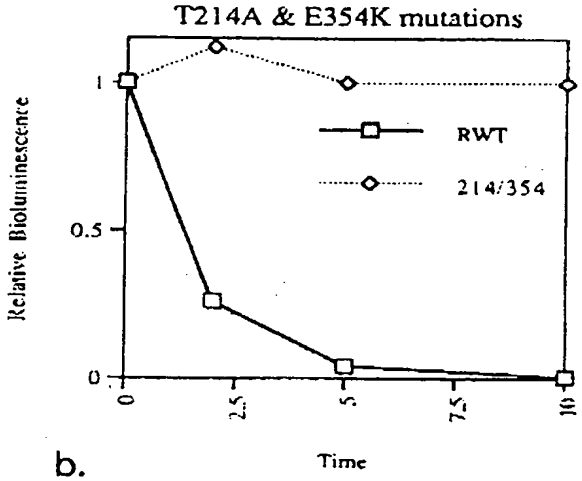

Thermostability at 40 °C of E345K/T214A/I232A and E354K/A215L mutant luciferases
% initial activity
110
100
90
80
70
60
50
40
30
20
10
0
0
2
4
6
8
10
12
14
16
time (min)
E345K/T214A/I232A
E354K/A215L

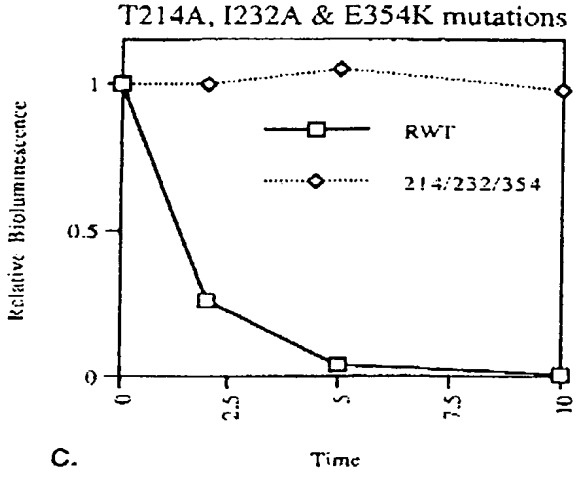
FIGURE 3



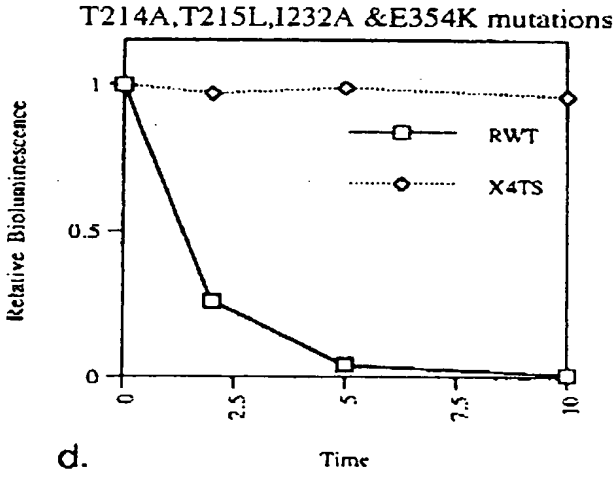
a.



b.

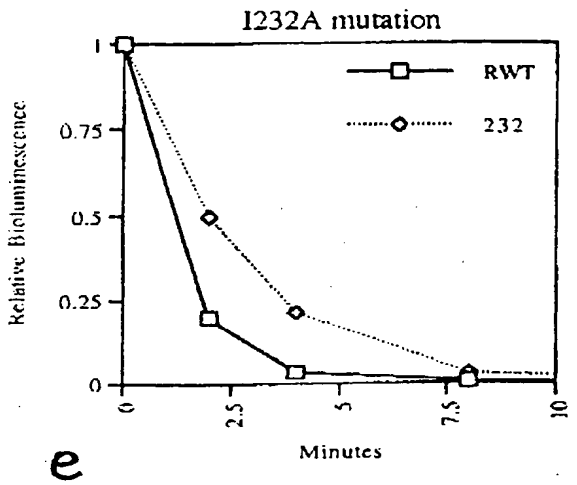


c.



d.

Figure 3 (cont'd)



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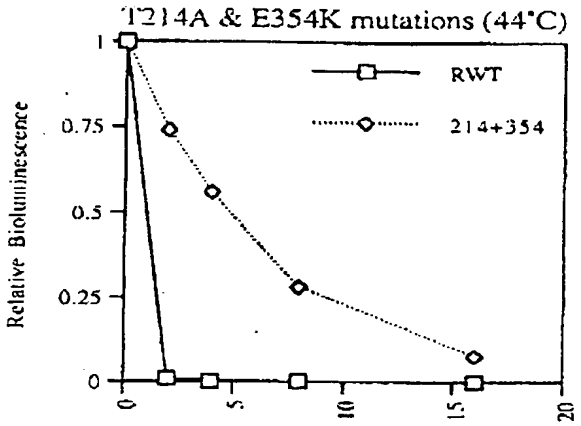

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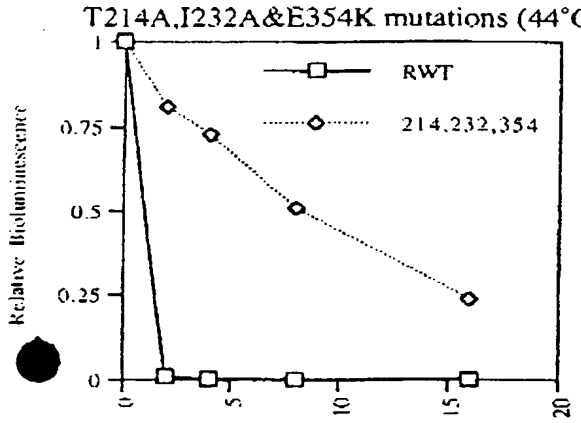

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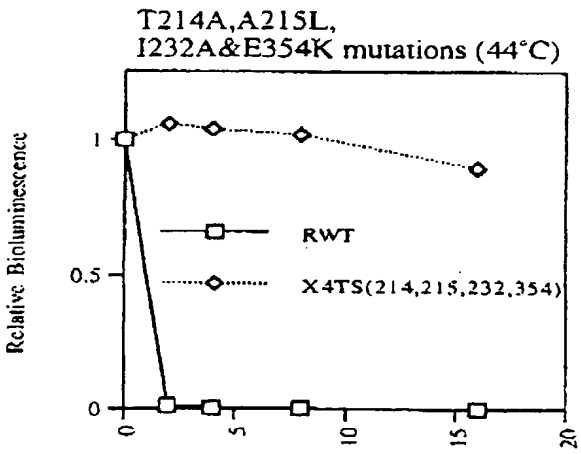

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